Isolation and characterization of endosymbiotic bacteria from copper contaminated soils in Chile

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Summary

Legume endosymbiotic bacteria indigenous of copper (Cu)-contaminated soils from Chile have been isolated using pea (*Pisum sativum*), bean (*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*) as trap host plants. Highly contaminated soils only produced nodules in certain legume hosts, whereas nodulation was observed in the three legume hosts when inoculated with soils containing a low Cu concentration. A collection of 362 strains was isolated, and their levels of Cu resistance were tested in media supplemented with increasing metal concentrations and in disk diffusion assays. By these two approaches, 84 strains displaying levels of Cu resistance higher than those exhibited by the corresponding reference strains were selected. The most resistant strains isolated from alfalfa and bean nodules grew normally at 3 mM and 2.5 mM $CuSO_4$ and were obtained from two different highly contaminated soils. Strains nodulating pea plants showed similar levels of resistance to Cu (2-2.5 mM $CuSO_4$) and were isolated from low-contaminated soils. Our data suggest a reduction of microbial diversity in agricultural Cu-contaminated soils from Chile.

Introduction

The widespread use of Cu in bactericides and fungicides lead to the contamination of agricultural soils mainly due to its accumulation to toxic levels in the surface soil layer. It is known that Cu and other heavy metals have a negative effect on legume endosymbiotic bacteria and on nitrogen fixation process (Laguerre *et al*., 2006), but the level of tolerance to Cu is highly variable among species and strains (Lakzian *et al*., 2002). The Cu extraction processes and wastes affect many agricultural soils in Chile. This situation raised the interest for studies of the biodiversity of legume endosymbiotic bacteria in these soils, and for the isolation of bacteria highly resistance to this heavy metal, in order to identify bacterial mechanisms involved in Cu homeostasis and bacterial survival in contaminated soils.

Materials and Methods

Pea (*Pisum sativum*), bean (*Phaseolus vulgaris)* and alfalfa (*Medicago sativa*) seedlings were inoculated with soils suspensions. Nodules were surfaced-sterilized, crushed and spread in YMB and R minimal media. Cu resistance of strains was analyzed by growing in plates of TY medium with increasing concentrations of $CuSO_4$. For disk diffusion tests (Bauer *et al.,* 1966), exponential cultures were inoculated in TY agar medium and disks soaked in different $CuSO_4$ concentrations. The inhibition zone was measured after 48 hours of incubation. Phylogenetic analysis was carried out with the 16S rDNA sequences of isolated strains. DNA sequences were optimally aligned using the CLUSTALX program. Neighbor-joining matrixes and trees were generated employing the MEGA 4.1 and the phylogenetic web server at www.phylogeny.fr.

Results and Discussion

The aim of this work was to study de diversity of *Rhizobium* species in Cu-contaminated soils of Chile and the identification of rhizobial strains with increased resistance to Cu. To do this, nine soils were prospected from the III, IV and VI regions. Among them three soils, designated as 7-12, 9-11 and Copiapó, contained the highest concentration of Cu and were classified as highly contaminated. Pea, bean and alfalfa plants were used as trap legume hosts to isolate the rhizobia indigenous population. After three weeks of soil inoculation, root

nodulation was checked. In general all hosts inoculated with the less contaminated soils showed a normal nodulation, whereas plants inoculated with suspensions of highly contaminated soils failed to nodulate certain hosts. In fact, no nodulation was observed in any host inoculated with the Copiapo soil sample, the most contaminated soil. A collection of 362 isolates from pea, bean and alfalfa nodules was obtained. Cu resistance was analyzed by growing isolated strains in plates containing concentrations of Cu ranging from 0.5 to 3.5 mM $CuSO_4$ and by disk diffusion tests (Figure). By these approaches 84 strains displaying levels of Cu resistance higher than those exhibited by the corresponding reference strains were selected. The most resistant strains isolated from alfalfa and bean nodules grew normally at 3 mM and 2.5 $CuSO_4$ and were obtained from highly contaminated soils 7-12 and 9-1. All strains obtained from pea nodules showed similar levels of resistance to Cu (2-2.5 mM $CuSO_4$) and were isolated from non-contaminated soils.

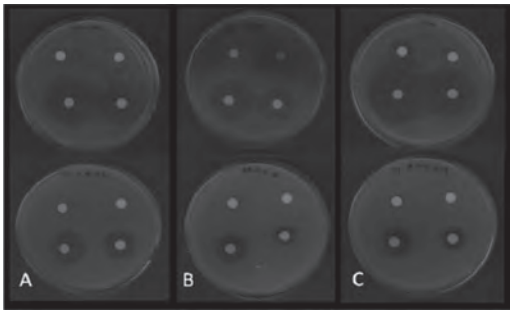

Figure. Analysis of Cu resistance of endosymbiotic bacteria isolated from pea (strain G.M.D.4, Panel **A**, bottom), bean (strain J.9-11.C.14, Panel **B**, bottom), and alfalfa (strain A.7-12.C.19, Panel **C**, bottom) nodules by disk diffusion tests. Plates in the top are inoculated with the respective control strains: *R. leguminosarum* strain UPM791 (Panel **A**), *R. etli* CFN42 (Panel **B**) and *S. meliloti* 1021(Panel **C**). Each plate contains disk soaked in water, 100 mM, 500 mM, 1 M $CuSO_4$.

According to the phylogenetic characterization based on 16S rRNA sequences, the pea isolates are grouped together forming a monophyletic group together with the corresponding strains of *R. leguminosarum* bv. *viciae*, which agrees with the results of the tests of nodulation. The sequences of the bean strains showed a high sequence similarity with the corresponding of *R. etli* and *R. leguminosarum* bv. *phaseoli* strains. Sequences belonging to the alfalfa isolates grouped together with sequences coding for 16S rRNA in strains of *S. meliloti*, and *Sinorhizobium* sp. Our data suggest a reduction of microbial diversity in agricultural Cu-contaminated soils from Chile.

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